Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast

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Background: In animal cells, recruitment of phosphatidylinositol 3-kinase by growth factor receptors generates 3-phosphoinositides, which stimulate 3-phosphoinositide-dependent protein kinase-1 (PDK1). Activated PDK1 then phosphorylates and activates downstream protein kinases, including protein kinase B (PKB)/c-Akt, p70 S6 kinase, PKC isoforms, and serum- and glucocorticoid-inducible kinase (SGK), thereby eliciting physiological responses.

Results: We found that two previously uncharacterised genes of *Saccharomyces cerevisiae*, which we term *PKH1* and *PKH2*, encode protein kinases with catalytic domains closely resembling those of human and *Drosophila* PDK1. Both Pkh1 and Pkh2 were essential for cell viability. Expression of human PDK1 in otherwise inviable *pkh1Δ pkh2Δ* cells permitted growth. In addition, the yeast *YPK1* and *YKR2* genes were found to encode protein kinases each with a catalytic domain closely resembling that of SGK; both Ypk1 and Ykr2 were also essential for viability. Otherwise inviable *ypk1Δ ykr2Δ* cells were fully rescued by expression of rat SGK, but not mouse PKB or rat p70 S6 kinase. Purified Pkh1 activated mammalian SGK and PKBα *in vitro* by phosphorylating the same residue as PDK1. Pkh1 activated purified Ypk1 by phosphorylating the equivalent residue (Thr504) and was required for maximal Ypk1 phosphorylation *in vivo*. Unlike PKB, activation of Ypk1 and SGK by Pkh1 did not require phosphatidylinositol 3,4,5-trisphosphate, consistent with the absence of pleckstrin homology domains in these proteins. The phosphorylation consensus sequence for Ypk1 was similar to that for PKBα and SGK.

Conclusions: Pkh1 and Pkh2 function similarly to PDK1, and Ypk1 and Ykr2 to SGK. As in animal cells, these two groups of yeast kinases constitute two tiers of a signalling cascade required for yeast cell growth.

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Background

Receptor-mediated activation of phosphatidylinositol (PI) 3-kinase in animal cells stimulates a recently identified 3-phosphoinositide-dependent protein kinase, termed PDK1 (for review, see [1,2]). This enzyme was first identified [3,4] by its ability to activate protein kinase B (PKB) [5], also called RAC (for related to PKA and PKC) kinase [6], and known also as the cellular homologue (c-Akt) of a retroviral oncoprotein, v-Akt [7]. PDK1 activates all known isoforms of PKB/c-Akt by phosphorylating a threonine residue in a conserved sequence motif (**Thr**–Phe–Cys–Gly–Thr–X–Glu–Tyr, where the bold Thr represents the phosphorylated residue and X represents any amino acid) located within the 'activation loop' of the catalytic domain, situated between the conserved protein kinase subdomains VII and VIII [8]. This residue corresponds to Thr308 in PKBα, Thr309 in PKBβ, and Thr305 in PKBγ [9].

PDK1 is a monomeric 63 kDa enzyme comprising an amino-terminal catalytic domain and a carboxy-terminal extension containing a pleckstrin homology (PH) domain that binds PI 3,4,5-trisphosphate ($PI(3,4,5)P_3$) or PI 3,4-bisphosphate ($PI(3,4)P_2$) or, more weakly, $PI(4,5)P_2$ [10,11]. $PI(3,4,5)P_3$ is generated from $PI(4,5)P_2$ by PI 3-kinase, and can be converted to $PI(3,4)P_2$ or $PI(4,5)P_2$ by different classes of 5- and 3-phosphoinositide phosphatases [12,13]. Like PDK1, PKB contains a PH domain specific for $PI(3,4,5)P_3$ and $PI(3,4)P_2$ [14,15], although in PKB it is located at the amino terminus. The PH domain of PKB blocks access to the phosphorylation site in the carboxy-terminal catalytic domain and this inhibition is relieved upon binding of lipids to the PH domain. Correspondingly, PKB is only activated by PDK1 *in vitro* in the presence of lipid vesicles containing $PI(3,4,5)P_3$ or $PI(3,4)P_2$ (for review, see [16,17]).

Since its discovery, PDK1 has been shown to phosphorylate other classes of protein kinases *in vitro* and cumulative evidence indicates that PDK1 is responsible for activating these enzymes *in vivo*. Thus, PDK1 may serve as a central

integrator for signalling events from receptors that stimulate PI 3-kinase. Additional PDK1 targets elucidated to date include: p70 S6 kinase [18,19]; at least two PKC isoforms [20,21]; the catalytic subunit of cAMP-dependent protein kinase (PKA) [22]; and, most recently, serum- and glucocorticoid-inducible kinase (SGK) [23]. All of these enzymes contain in their activation loop the conserved phosphorylation sequence first found in PKB/c-Akt and are phosphorylated by PDK1 at the corresponding threonine residue. Hence, this motif has been termed the PDK1 site. Full activation of PKB, however, also requires phosphorylation at a second site, Ser473, situated in a hydrophobic motif (Phe–X–X–Ar–**Ser/Thr**–Ar, where the bold residue is the phosphorylated amino acid and Ar represents an aromatic residue) towards the carboxyl terminus [24]. Phosphorylation at this second site in PKB *in vivo* is prevented by inhibitors of PI 3-kinase [24]. This second motif is also conserved in the other PDK1-activated protein kinases listed above (except for PKA) and lies at a roughly equivalent position carboxy-terminal to the PDK1 site [20,25,26]. Neither purified nor recombinant PDK1 phosphorylates this second site [3,27], suggesting that this event requires either modification of the activity of PDK1 or a distinct enzyme, provisionally termed PDK2.

If PDK1 and its target protein kinases perform functions vital to signalling in all eukaryotic cells, then these molecules should be evolutionarily conserved. We have shown previously that a PDK1-like enzyme is present in the fruit fly, *Drosophila melanogaster* [27]. Here, we demonstrate both genetically *in vivo* and biochemically *in vitro* that PDK1-like and SGK-like protein kinases are present in budding yeast, *Saccharomyces cerevisiae*, and that these kinases are essential for cell growth and viability. Additional findings suggest that, as in animal cells, the PDK1-like enzymes are likely to play a role in activating other classes of protein kinases, in addition to the SGK-like enzymes.

Results

PKH1 and *PKH2* encode homologues of mammalian PDK1

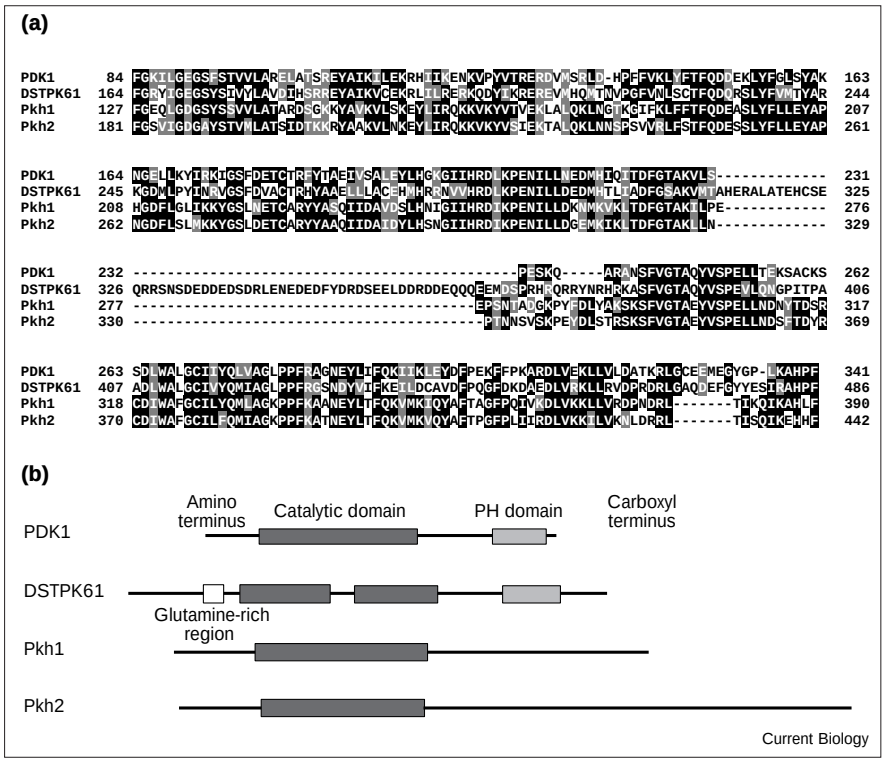
The *S. cerevisiae* genome contains two, previously uncharacterised, open reading frames (YDR490c and YOL100w) that encode protein kinases whose catalytic domains are 72% identical and share 50% identity with either human or *Drosophila* PDK1 (Figure 1a). These loci were designated *PKH1* and *PKH2*, respectively (for PKB-activating kinase homologues 1 and 2). *PKH1* is located on the right arm of chromosome IV [28] and encodes a 766-residue protein (86 kDa); *PKH2* is situated on the left arm of chromosome XV [29] and encodes a 1,081-residue protein (121 kDa). Pkh1 and Pkh2 contain both amino-terminal and carboxy-terminal extensions to the catalytic domain (Figure 1b) and are much less similar to each other (only 27% identity) in these regions. These regions display no apparent similarity with the non-catalytic regions of PDK1 or with other known proteins. In particular, Pkh1 and Pkh2 lack any obvious PH domain, unlike human and *Drosophila* PDK1 which have a PH domain at their carboxyl terminus.

PKH1 and *PKH2* are essential genes that are functionally redundant

To determine whether the genes *PKH1* and *PKH2* encode expressed proteins, the effect of loss-of-function mutations in these loci was examined. Each open reading frame was deleted and replaced with a selectable marker (*TRP1* for *PKH1* and *HIS3* for *PKH2*). The resulting alleles, *pkh1Δ::TRP1* and *pkh2Δ::HIS3*, were used to replace the normal chromosomal loci by homologous recombination. Both a haploid *pkh1Δ::TRP1* mutant strain (AC301) and a haploid *pkh2Δ::HIS3* mutant strain (AC303) grew normally and indistinguishably from congenic *PKH1*$^+$ and *PKH2*$^+$ haploids isolated from the same tetrad. Also, *pkh1Δ* and *pkh2Δ* single mutants displayed no apparent phenotype when challenged by high concentrations of salt or caffeine, various carbon sources, different temperatures, or when subjected to heat shock. To determine whether *PKH1* and *PKH2* have a common function, the AC301 strain was crossed with the AC303 strain. Upon sporulation of the resulting doubly heterozygous diploid (AC306), the majority of the 30 tetrads dissected yielded three viable spores and one non-viable spore. The viable spores were analysed both by plating on appropriate selective media and by PCR. None of the viable haploid cells were Trp$^+$ and His$^+$, and none carried both the *pkh1Δ* and the *pkh2Δ* mutations. Microscopic observation of the non-viable spores revealed that most germinated and underwent 2–3 cycles of cell division before ceasing to grow. Hence, *pkh1Δ pkh2Δ* double mutants are inviable, indicating that *PKH1* and *PKH2* encode genes that are functionally redundant and share some role that is essential for cell growth and survival.

To confirm that the lethality of *pkh1Δ pkh2Δ* cells is due solely to the absence of *PKH1* or *PKH2* function, the doubly heterozygous diploid strain AC306 was transformed with either YEplac195-*PKH1*, a *URA3*-marked plasmid expressing *PKH1* from its own promoter, or the empty vector (YEplac195), and the resulting Ura$^+$ transformants were subjected to sporulation and tetrad dissection. Many Trp$^+$His$^+$ and Ura$^+$ spore clones were obtained from the diploid strain transformed with YEp195-*PKH1*, but not from that transformed with empty vector (data not shown). Thus, the *pkh1Δ pkh2Δ* double mutant was able to survive if *PKH1* expression was restored. Likewise, when AC306 was transformed with a *URA3*-marked plasmid (pYES2) expressing either *PKH1* (Figure 2a) or *PKH2* (data not shown) from the *GAL1* promoter, it was possible to obtain viable *pkh1Δ pkh2Δ* spores, even when the cells were propagated on glucose (presumably because these constructs are not efficiently repressed on this carbon source [30]). Most significantly, when the *pkh1Δ pkh2Δ* cells harbouring

Figure 1



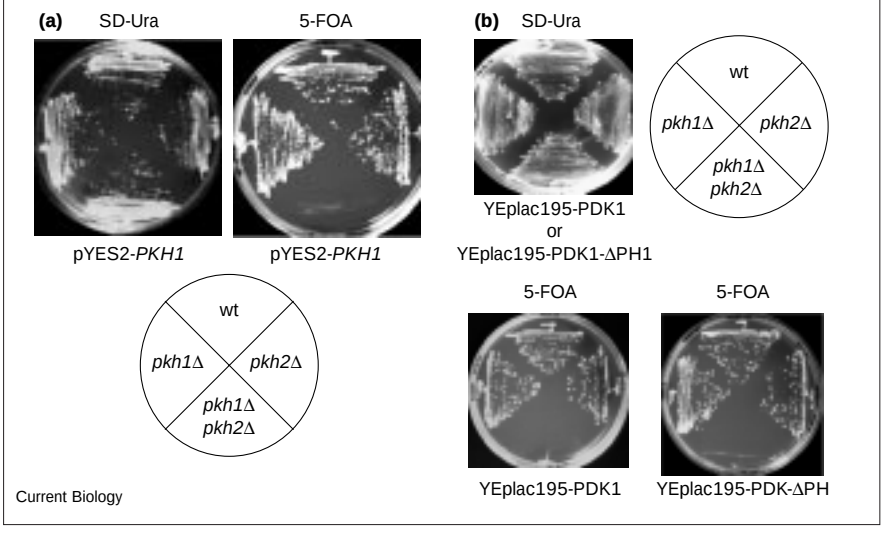
Comparison of the primary structures of Pkh1 and Pkh2 with PDK1. (a) Alignment of the deduced amino-acid sequences of yeast Pkh1 and Pkh2 with the catalytic domains of human PDK1 and its *Drosophila* homologue, DSTPK61 [27], carried out using the CLUSTAL W program. Identical residues are denoted by white letters on a black background, and similar residues by white letters on a grey background. Dashes represent gaps introduced in the sequences to optimise the alignment. Five independent *PKH1* clones that we generated by PCR all differed from the sequence deposited in the *Saccharomyces* Genome Database, indicating that Phe187 (nucleotide sequence TTC) should actually be an isoleucine residue (ATC). (b) Schematic diagrams of the structures of the PDK1-related proteins. Dark grey boxes indicate the catalytic domain in each protein kinase and light grey boxes indicate PH domains.

pYES2-*PKH1* (Figure 2a) or pYES2-*PKH2* (data not shown) were plated on medium containing 5-fluoro-orotic acid (5-FOA), which selects for cells that lack a functional *URA3* gene [31] and, hence, for loss of the *URA3*-marked plasmid, the *pkh1Δ pkh2Δ* cells were no longer capable of growing (Figure 2a).

Human PDK1 is a functional homologue of Pkh1 and Pkh2

To determine whether Pkh1 and Pkh2 are similar to PDK1 in function, as well as in sequence, the AC306 strain was transformed with YEplac195 plasmids containing genes encoding either full-length human PDK1 or human PDK1 lacking the carboxy-terminal PH domain

Figure 2



Loss of both *PKH1* and *PKH2* causes inviability and either intact human PDK1 or PDK1-ΔPH can restore viability to *pkh1Δ pkh2Δ* cells. (a) Four spores (wild type (wt); *pkh1Δ*; *pkh2Δ*; and *pkh1Δ pkh2Δ*) derived from a tetratype ascus of diploid strain AC306 that had been transformed with plasmid pYES2-*PKH1* (which is marked with *URA3*) were plated either on medium lacking uracil (SD-Ura) to select for the presence of the plasmid, or on medium containing 5-FOA to select for loss of the plasmid [31]. (b) Four spores, as in (a), derived from a tetratype ascus of strain AC306 that was transformed with either YEplac195-PDK1 or YEplac195-PDK1-ΔPH (which are marked with *URA3*), as indicated, were plated either on medium lacking uracil (SD-Ura) to select for the presence of the plasmids (only one representative plate is shown), or on medium containing 5-FOA to select for loss of the plasmids.

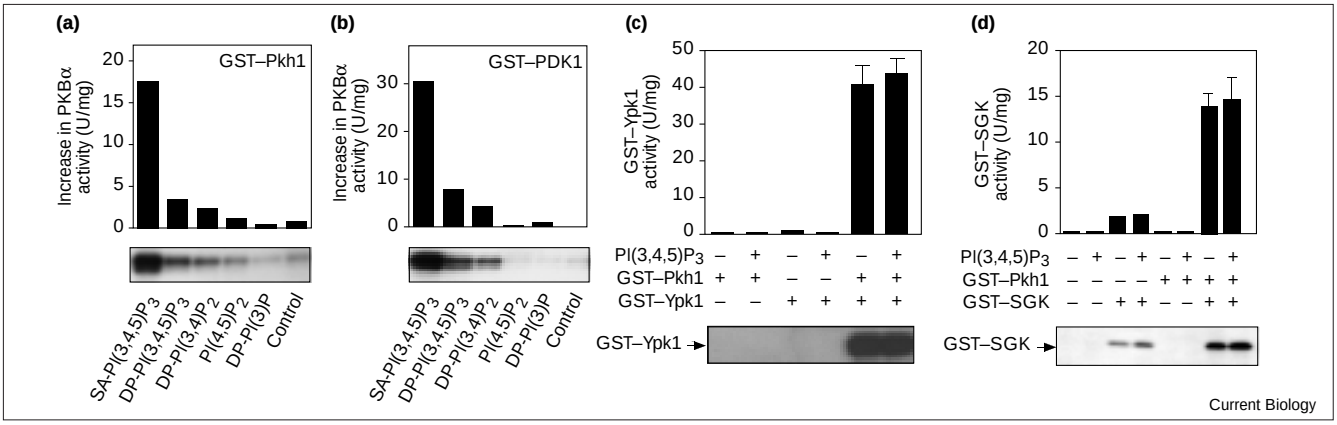
(PDK1-ΔPH) under control of the authentic *PKH1* promoter. Expression of either PDK1 or PDK1-ΔPH from this vector permitted the recovery of viable *pkh1Δ pkh2Δ* spores. Moreover, selection against these *URA3*-marked plasmids by plating on 5-FOA-containing medium prevented the growth of the *pkh1Δ pkh2Δ* double mutants, but not a *pkh1Δ* or a *pkh2Δ* single mutant carrying the same plasmids (Figure 2b). Viable *pkhΔ pkh2Δ* double mutant spores could also be recovered when AC306 was transformed with pYES2 plasmids containing genes encoding either PDK1 or PDK1-ΔPH driven by the *GAL1* promoter, even when the cells were germinated and grown on glucose-containing medium (data not shown). These results demonstrate that the catalytic domain of human PDK1 is able to phosphorylate the same essential substrates in yeast cells as Pkh1 and Pkh2.

Purification and characterisation of Pkh1 kinase activity

Pkh1 was expressed as a glutathione-S-transferase (GST) fusion protein in human 293 cells and purified from cell lysates by affinity chromatography on glutathione–Sepharose. This process yielded a single protein that migrated with the expected molecular mass (112 kDa) by SDS–PAGE. A catalytically inactive (kinase-dead) mutant, which contains a substitution of the conserved residue (alanine for aspartic acid) critical for binding to the Mg^{2+}–ATP substrate, Pkh1(D267A), was also expressed and purified, and served as a control. GST–Pkh1 activity was measured via its ability to phosphorylate and activate GST–PKBα, a known substrate of human PDK1. The phosphorylation of PKBα was assessed by the incorporation of label in reactions containing [γ-^{32}P]ATP. The activity of PKBα was followed via its ability, after incubation with GST–Pkh1, to phosphorylate a specific peptide substrate (crosstide) [32]. Yeast Pkh1 was able to activate human PKBα (Figure 3a), provided that lipid vesicles containing PI(3,4,5)P_3 or PI(3,4)P_2 were present. The Pkh1-dependent phosphorylation of PKBα correlated well with the degree of activation observed. No activation or phosphorylation of PKBα was observed if PI(3,4,5)P_3 or PI(3,4)P_2 were omitted, or if these lipids were replaced by PI(4,5)P_2 or PI(3)P (Figure 3a). As observed with human PDK1 (Figure 3b), Pkh1 phosphorylated and activated PKBα more efficiently in the presence of the naturally occurring stearoyl–arachidonyl derivative of PI(3,4,5)P_3 than in the presence of the dipalmitoyl derivative. GST–Pkh1(D267A) did not activate or phosphorylate PKBα under any condition tested (data not shown).

To determine the residue phosphorylated by Pkh1, GST–PKBα was phosphorylated to completion by prolonged incubation in the presence of [γ-^{32}P]ATP, cleaved with trypsin, and the resulting digest resolved by high performance liquid chromatography (HPLC) on an RP-C_{18} column. Only one major phosphopeptide was obtained (data not shown). Elution of this species was congruent with that of the phosphopeptide (residues 308–328) obtained by tryptic digestion of PDK1-phosphorylated PKBα. No ^{32}P-labelled material eluted at the position corresponding to the PKBα peptide that contains Ser473. The peptide labelled by Pkh1 contained phosphothreonine (and no other phosphoamino acid) and all of the

Figure 3



Phosphorylation and activation of human PKBα by yeast Pkh1 and human PDK1, and phosphorylation and activation of yeast Ypk1 and human SGK by yeast Pkh1. **(a,b)** Purified GST–PKBα was incubated for 30 min at 30°C with either (a) GST–Pkh1 or (b) GST–PDK1 in the presence of 100 μM ATP and phospholipid vesicles containing 100 μM phosphatidylcholine and 100 μM phosphatidylserine together with the various PI lipids indicated (where SA denotes stearoyl–arachidonyl derivatives and DP denotes dipalmitoyl derivatives), all at a final concentration of 10 μM. The phosphorylation of GST–PKBα and the increase in its specific activity (relative to control incubations in which GST–Pkh1 or GST–PDK1 were omitted) were determined (see Materials and methods). **(c,d)** Either (c) GST–Ypk1 or (d) GST-SGK were incubated for 30 min at 30°C with GST–Pkh1, as indicated, in the presence of 100 μM ATP, with or without phospholipid vesicles containing 100 μM phosphatidylcholine, 100 μM phosphatidylserine and 10 μM of SA-PI(3,4,5)P_3, collectively termed PI(3,4,5)P_3. Reactions were terminated and the degree of activation and phosphorylation was determined.

radioactivity was released after one cycle of Edman degradation (data not shown). This analysis establishes that yeast Pkh1 phosphorylates PKBα at Thr308.

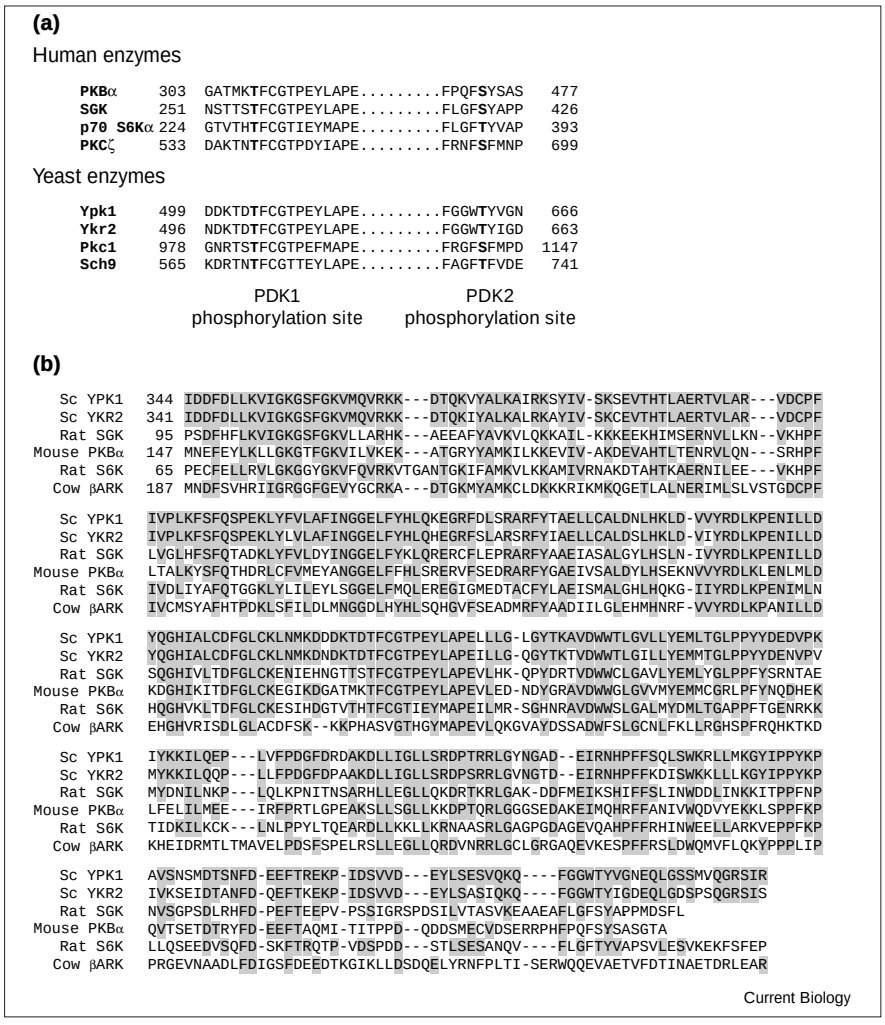
YPK1 and *YKR2* encode homologues of mammalian SGK

PKBα is one founding member of the so-called AGC subfamily of protein kinases, several of which possess the conserved PDK1 phosphorylation site in their activation loop and a distal PDK2 phosphorylation site (Figure 4a). There are four previously characterised protein kinases in *S. cerevisiae* that possess both motifs (Figure 4a), suggesting that one or more might be physiological substrates for Pkh1 and/or Pkh2. These four protein kinases are the products of the *YPK1* [33], *YKR2/YPK2* [34,35], *SCH9* [36], and *PKC1* [37] genes. Ypk1 and Ykr2 are 88% identical in their catalytic domains and have extensive similarities across their amino-terminal and carboxy-terminal extensions (Figure 4b). Among mammalian protein kinases, the catalytic domains of Ypk1 and Ykr2 share greatest similarity to SGK (55% identity), PKB/c-Akt (52% identity), p70 S6 kinase (50% identity) and βARK (38% identity; Figure 4b). Although βARK is considered to be an AGC family member [38], it lacks the PDK1 and PDK2 motifs.

YPK1 and *YKR2* are essential genes that are functionally redundant

Cells lacking either Ypk1 or Ykr2 are viable, whereas cells lacking both Ypk1 and Ykr2 are inviable [35]. We established conditions for testing the ability of potential mammalian homologues to rescue this inviability. For this purpose, otherwise isogenic *ypk1Δ::HIS3* and *ykr2Δ::TRP1* haploid strains (YES5 and YES1, respectively) were crossed and the resulting diploid strain (YES7) was transformed with a *LEU2*-marked plasmid expressing the *YKR2* gene under the tight control of the *GAL1* promoter. When subjected to sporulation and tetrad dissection on galactose-containing medium, most of the tetrads yielded four viable spores, and $His^+Trp^+Leu^+$ isolates were readily

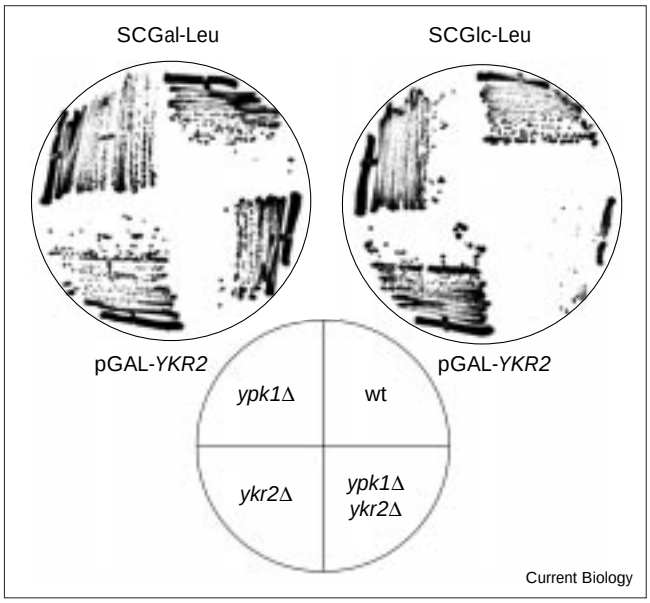
Figure 4



Consensus sites for PDK1-dependent and PDK2-dependent phosphorylation, and comparison of the primary structures of Ypk1 and Ykr2 with their closest mammalian homologues. (a) Conserved motifs predicted to be phosphorylated by PDK1-like and PDK2-like enzymes are indicated with the residue phosphorylated shown in bold type. In all cases, the putative PDK2 phosphorylation site is located 157–166 residues carboxy-terminal to the PDK1 phosphorylation site. GenBank accession numbers: PKBα, X65687; SGK, Y10032; p70 S6Kα, M60725; PKCζ, L07032; Ypk1, P12688; Ykr2, P18961; Pkc1, M32491; and Sch9, U00029. (b) Alignment of Ypk1 and Ykr2 to each other and to mammalian SGK, PKBα, p70 S6 kinase (S6K) and βARK: those residues in Ypk1 that are conserved in at least one of the other kinases are indicated by black letters on a grey background.

recovered. The *ypk1Δ ykr2Δ* double mutants carrying pGAL-*YKR2* could be maintained on galactose medium, but failed to grow when streaked onto glucose medium, whereas otherwise isogenic wild-type cells or *ypk1Δ* and *ykr2Δ* single mutants carrying the same plasmid grew well on glucose (Figure 5). These results confirm that Ypk1 and Ykr2 share some role that is essential for cell growth and survival.

Figure 5



Expression of either Ypk1 or Ykr2 is required for viability. Strain YES7, transformed with the *LEU2*-marked plasmid, pGAL-*YKR2*, was sporulated on medium lacking leucine and containing galactose to select for the presence of the plasmid and to induce expression of *YKR2* from the plasmid. The four spores of a tetratype ascus (wild type (wt); *ypk1Δ*; *ykr2Δ*; *ypk1Δ ykr2Δ*) derived from this diploid were recovered on this medium (SCGal-Leu) and then subsequently streaked on the same medium containing glucose as the carbon source (SCGlc-Leu) to repress expression of the plasmid-borne *YKR2* gene.

SGK is a functional homologue of Ypk1 and Ykr2

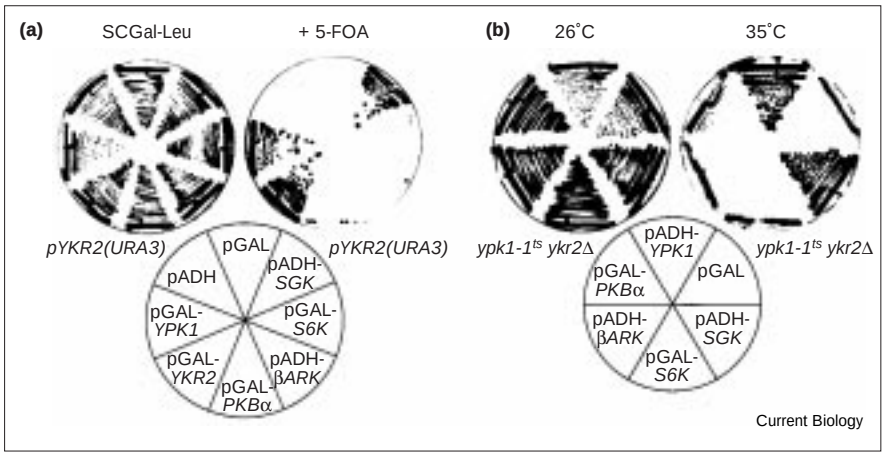
To construct a strain for conveniently testing complementation of the *ypk1Δ ykr2Δ* double mutant by mammalian protein kinases, the diploid YES7 strain was transformed with a *URA3*-marked low-copy-number (*CEN*) plasmid expressing *YKR2* from its own promoter, and a haploid *ypk1Δ ykr2Δ* double mutant strain maintained by expression of *YKR2* from the plasmid (strain YPT28) was recovered. The YPT28 strain was then transformed with either empty *LEU2*-marked, high-copy-number (2 μm DNA) vectors (pAD4M or YEp351GAL) or the same vectors expressing (from either the *GAL1* promoter or the constitutive *ADH1* promoter [39]) *YPK1*, *YKR2*, or cDNAs encoding rat SGK, mouse PKB/c-AKT, rat p70 S6 kinase, or bovine βARK. All of these strains are able to grow on galactose medium due to the presence of the plasmid expressing *YKR2*, which also demonstrated that expression of none of the heterologous protein kinases tested was deleterious to yeast cell growth (Figure 6a). In contrast, when plated on the same medium containing 5-FOA, thereby demanding loss of the p*YKR2*(*URA3*) plasmid, *ypk1Δ ykr2Δ* cells carrying the empty vectors were unable to grow, whereas those harbouring plasmids expressing either *YPK1* or *YKR2* remained viable, as expected.

Of the four mammalian cDNAs tested, only SGK displayed efficient complementation by permitting growth on 5-FOA (Figure 6a). Weak complementation by PKB was reproducibly observed, in that a small percentage of the colonies were able to survive (possibly cells expressing exceedingly high levels of PKB due to elevated copy number of the 2 μm DNA plasmid resulting from its poor segregation efficiency [40]). Each mammalian protein kinase was produced at a readily detectable level in yeast (grown on SCGal-Leu), as judged by immunoblotting with appropriate antibodies, and was active, as judged by assaying extracts of the yeast cells with appropriate specific substrates (data not shown); hence, the failure of p70 S6 kinase and βARK to complement was not due to their lack of expression. Because p70 S6 kinase is under such complex regulation in animal cells [41,42], various amino-terminal and carboxy-terminal truncations [43] were also tested in the same way; although all were expressed, none were able to complement (data not shown). To confirm these results by an independent method, the same plasmids were introduced into a yeast strain (YPT40) that displays temperature-conditional growth because it carries a null mutation in *YKR2* (*ykr2Δ*) and a temperature-sensitive (ts) mutation in *YPK1* (*ypk1-1^{ts}*). All of the transformants were able to grow at the permissive temperature (26°C). However, the strain carrying an empty vector (YEp351GAL) was unable to survive at the restrictive temperature (35°C), although the strain expressing *YPK1* from the same vector grew well (Figure 6b). As observed before, the same cells expressing SGK were able to grow well at 35°C. Cells expressing PKB were able to grow weakly, in that microcolonies were observed outside of the heavy initial streak. In contrast, cells expressing p70 S6 kinase or βARK did not grow (Figure 6b).

Ypk1 and mammalian SGK are efficient substrates for Pkh1

On the basis of the above observations, Ypk1 (and/or Ykr2) should be physiological substrates of Pkh1 (and/or Pkh2). Likewise, as PDK1 is able to phosphorylate and activate SGK [23], yeast Pkh1 (and/or Pkh2) should be able to phosphorylate and activate mammalian SGK. To test these predictions, Ypk1 and SGK (lacking its amino-terminal 60 amino acids) were expressed as GST fusion proteins in 293 cells and purified. Each purified protein yielded a single band following SDS–PAGE that had the expected molecular mass (data not shown). In the absence of any other factor, purified GST–Ypk1 displayed no

Figure 6



SGK rescues the inviability of *ypk1Δ ykr2Δ* cells. (a) Strain YPT28 carrying p*YKR2*, a *URA3*-marked plasmid that expresses *YKR2* from its authentic promoter, was generated and transformed with either empty *LEU2*-marked vectors pAD4M (pADH) or YEp351GAL (pGAL) or the same vectors expressing from either the *ADH1* or the *GAL1* promoters, as indicated, either *YPK1*, *YKR2*, *SGK*, PKBα, p70 S6 kinase (S6K) or βARK. The resulting transformants were then streaked onto medium selective for the presence of both plasmids (SCGal-Leu) or onto the same medium containing 5-FOA, thereby demanding loss of the *URA3*-marked plasmid expressing *YKR2*. (b) Strain YPT40 (*ypk1-1^{ts} ykr2Δ*) was constructed, transformed with the indicated plasmids described in (a), then streaked on selective medium and incubated at either a permissive temperature (26°C) or a restrictive temperature (35°C).

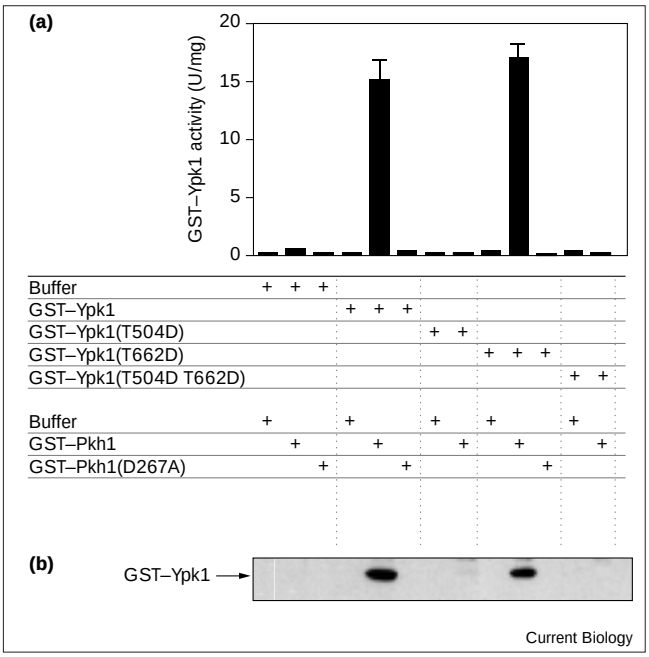
detectable activity towards the crosstide peptide substrate; however, after pre-incubation with purified GST–Pkh1, GST–Ypk1 was activated and catalysed a readily detectable level of incorporation into the substrate, in the presence or absence of lipid vesicles containing $PI(3,4,5)P_3$ (Figure 3c). Consistent with activation resulting from Pkh1-dependent phosphorylation, incorporation of label into GST–Ypk1 was readily detected when [γ-^{32}P]ATP was included in the reaction and was present exclusively as phosphothreonine (data not shown). A kinase-dead derivative, GST–Ypk1(D488A), was phosphorylated by GST–Pkh1 but, as expected, was not catalytically active (data not shown). Also, as predicted, GST–SGK was both phosphorylated and activated upon incubation *in vitro* with GST–Pkh1 (Figure 3d). Moreover, the site phosphorylated in SGK by Pkh1 was mapped to Thr256 (data not shown), the same site as that phosphorylated by PDK1 [23].

As one means to map the Pkh1 phosphorylation site in Ypk1, a GST–Ypk1(T504D) mutant, which contains a threonine to aspartic acid substitution at position 504 in the presumptive PDK1-like site (Figure 4a), was expressed in 293 cells and purified. GST–Ypk1(T504D) was not detectably phosphorylated by Pkh1 (Figure 7), consistent with the conclusion that Pkh1 phosphorylates Ypk1 at this residue. As GST–Ypk1(T504D) was neither constitutively active nor activated by Pkh1, the aspartic acid residue at this position cannot substitute for phosphothreonine in the activation of this protein kinase. A similar result has been obtained for SGK [23]. Ypk1 also contains a putative consensus site (Thr662) for PDK2 phosphorylation (Figure 4a). A GST–Ypk1 fusion protein containing a threonine to aspartic acid mutation of this site, GST–Ypk1(T662D), was expressed and purified from 293 cells and was also inactive; however, this protein was phosphorylated and activated by Pkh1 in a manner identical to GST–Ypk1 itself (Figure 7). When both Thr504 and Thr662 were mutated to aspartic acid residues, creating GST–Ypk1(T504D T662D), this mutant also displayed no detectable activity (before or after incubation with Mg^{2+}–ATP and GST–Pkh1), demonstrating that, unlike PKBα, mutation of these residues to aspartic acid residues cannot substitute for phosphothreonine to produce a constitutively active enzyme. A similar observation has been made for SGK [23].

Pkh1 acts on Ypk1 *in vivo*

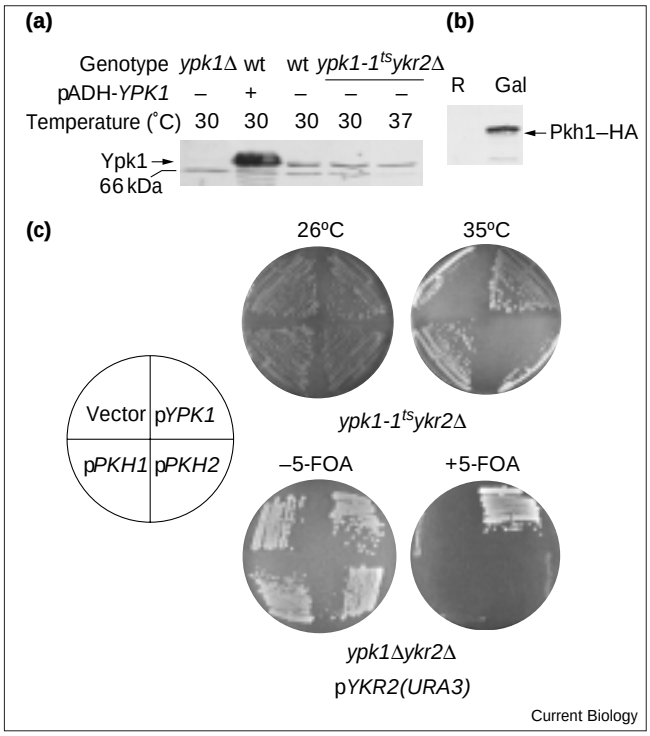
A functional Ypk1 derivative tagged at its carboxyl terminus with a c-Myc epitope was expressed in cells that were metabolically labelled with [^{32}P]PO_4^{3-}; the Ypk1 derivative was then immunoprecipitated with the anti-Myc antibody 9E10. When compared with the level of incorporation observed in control (*PKH1$^+$ PKH2$^+$*) cells, labelling of Ypk1 was reproducibly reduced (by 53 ± 20%) in *pkh1Δ* mutants, but not affected (within 10 ± 5% of control) in *pkh2Δ* mutants (see Supplementary material published with this paper on the internet). These results suggest that Pkh1 contributes to phosphorylation of Ypk1 *in vivo* and are consistent with the above findings *in vitro*. In agreement with these conclusions, overproduction of Pkh1 rescued the inviability of the *ypk1-1^{ts} ykr2Δ* strain at the non-permissive temperature, whereas Pkh2 overexpression did not (Figure 8). Moreover, as would be expected if this suppression arises from a direct physical interaction between Pkh1 and the mutant Ypk1 protein that persists at the restrictive temperature (Figure 8a), neither overproduced Pkh1 (Figure 8b) nor overproduced Pkh2 (data not shown) was able to rescue a *ypk1Δ ykr2Δ* strain, which lacks Ypk1 protein (Figure 8c).

Figure 7



Phosphorylation of Thr504 in Ypk1 is essential for its activation by Pkh1. (a) GST–Ypk1, GST–Ypk1(T504D), GST–Ypk1(T662D) or GST–Ypk1(T504D T662D) were incubated with either GST–Pkh1 or a catalytically inactive derivative, GST–Pkh1(D267A), as indicated, for 30 min at 30°C with ATP (100 μM). Reactions were terminated and the degree of activation assessed as indicated in the legend to Figure 3. (b) The reactions in (a) were carried out in the presence of 100 μM [γ-^{32}P]ATP and the products were analysed by SDS–PAGE followed by autoradiography to follow the amount of phosphate incorporation into GST–Ypk1 and its various derivatives, as described in the legend to Figure 3.

Figure 8



Overexpression of Pkh1 suppresses a temperature-sensitive Ypk1 mutant. (a) As judged by immunoblotting with rabbit polyclonal anti-Ypk1 antibodies, a polypeptide of the size predicted for Ypk1 was present in wild-type cells (wt), absent in a *ypk1Δ* mutant, overproduced in a cell expressing *YPK1* from a constitutive promoter (*ADH1*) on a multi-copy plasmid (pAD4M), as indicated, and persisted when strain YPT40 (*ypk1-1^{ts} ykr2Δ*) was grown in rich medium at 30°C and then shifted to the restrictive temperature (37°C) for 3 h. (b) A wild-type strain (W303-1A) carrying a plasmid (pGAL-*PKH1-HA*), that expresses from the *GAL1* promoter a version of Pkh1 tagged with an epitope derived from influenza virus haemagglutinin (HA), was grown in raffinose medium (R), and then a portion shifted to galactose medium (Gal) for 2 h, prior to analysis by SDS–PAGE and immunoblotting with a mouse anti-HA monoclonal antibody. (c) Strains YPT40 (*ypk1-1^{ts} ykr2Δ*) or YPT28 (*ypk1Δ ykr2Δ* p*YKR2*(*URA3*)) were transformed with the indicated *LEU2*-marked plasmids. The YPT40 derivatives were streaked on selective medium (SCGal-Leu) and incubated at the indicated temperatures for 3 days. The YPT28 derivatives were streaked on the same medium in the presence or absence of 5-FOA and incubated for 3 days at 30°C.

Substrate specificities of Ypk1, SGK and PKB are similar

PKBα phosphorylates substrates at the minimal consensus sequence Arg–X–Arg–X–X–**Ser**–Hyd, (where the bold Ser represents the phosphorylated amino acid and Hyd represents a bulky hydrophobic residue) [44]. Likewise, SGK requires arginine at the –5 and –3 positions (numbering with respect to Ser) for efficient phosphorylation, although the requirement for a large hydrophobic residue at +1 is less stringent [23]. Using peptide substrates, the substrate selectivity of Pkh1-activated Ypk1 was quite similar to that of PDK1-activated PKBα and PDK1-activated SGK (Table 1). For example, mutation of either of the arginine residues, even to lysine residues, drastically reduced phosphorylation by Ypk1.

Discussion

We have shown that *S. cerevisiae* contains protein kinases that are similar to mammalian PDK1 and SGK, in terms of sequence, physiological function *in vivo*, and biochemical specificity *in vitro*. We demonstrated that the *PKH1* and *PKH2* genes encode PDK1-like protein kinases. The function(s) of Pkh1 and Pkh2 must overlap because loss of either enzyme causes no obvious phenotype, whereas the deficiency of both proteins results in inviability. The catalytic domain of mammalian PDK1 alone was sufficient to rescue cells from this lethality. Consistent with this finding, purified Pkh1 phosphorylates and activates known substrates of mammalian PDK1, including PKBα and SGK, and phosphorylates the same residue in these substrates as that phosphorylated by PDK1 (Thr308 in PKBα and Thr256 in SGK).

Next, we demonstrated that the *YPK1* and *YKR2* gene products are SGK-like protein kinases. Although loss of Ykr2 produces no discernable phenotype, absence of Ypk1

Table 1

Substrate selectivity of yeast Ypk1 and mammalian PKBα.

	Peptide	Relative rate of phosphorylation (at 30 μM)		
		GST–Ypk1	GST–PKBα	GST–SGK†
1	GRPRTS**S**FAEG	(100)	(100)	100
2	RPRTS**S**F	92	139	154
3	K PRTS**S**F	11	6	2
4	RPK TS**S**F	5	30	49
5	RPRTSA F	0	0*	0
6	PRTS**S**F	0	0*	0
7	RPRTS**S**	0	2*	10
8	KKRNRTL**S**VA	133	157	ND
9	KK K NRTL**S**VA	28	13	ND
10	KKRN K TL**S**VA	14	25	ND
11	RPRTS**S**F	92	139	154
12	RPRTS**S**V	24	67	131
13	RPRTS**S**L	31	69	135
14	RPRTS**S**A	9	26	107
15	RPRTS**S**K	14	67*	146
16	RPRTS**S**E	6	21	84

*Data taken from [44]. †Data taken from [23]. ND, not determined. Bold residues indicate the phosphorylated site, and the underlined residues those that have been altered.

causes slower cell growth and removal of both Ykr2 and Ypk1 causes inviability [35,45], indicating that these enzymes have some functional redundancy. Expression of SGK, a close relative of PKBα that lacks an obvious amino-terminal PH domain, rescued this inviability efficiently. PKB itself was able to sustain the growth of the *ypk1Δ ykr2Δ* cells only very weakly. Indeed, PKB in yeast should exist in an inactive state because *S. cerevisiae* lacks the enzymatic machinery necessary to generate $PI(3,4,5)P_3$ or $PI(3,4)P_2$ [46–48]. This situation could explain why Pkh1, Pkh2, Ypk1 and Ykr2 lack discernible PH domains and why Pkh1-dependent activation of Ypk1 *in vitro* is not affected by the presence or absence of these 3-phosphoinositides. In fact, Pkh1 does not bind $PI(3,4,5)P_3$ under conditions where this phospholipid binds tightly to PDK1 and PKBα (R. Currie and C.P. Downes, personal communication). Therefore, our finding that Pkh1-mediated activation of PKBα only occurred in the presence of lipid vesicles containing $PI(3,4,5)P_3$ or $PI(3,4)P_2$ confirms that these 3-phosphoinositides exert their effect by interacting with the PH domain of PKBα and thereby enhancing the accessibility of its PDK1 site. Signals that activate Pkh1 and Pkh2 or recruit these enzymes to particular subcellular locations are not yet known. Likewise, signals that promote or prevent encounter of Ypk1 and Ykr2 with Pkh1 and Pkh2 are also unknown.

Other protein kinases highly related to Ypk1 and Ykr2 (including p70 S6 kinase and βARK) did not support growth of cells deficient in both Ypk1 and Ykr2, even though these kinases were expressed in an active form in yeast. Thus, functional complementation strongly argues that SGK is the most similar kinase to Ypk1 and Ykr2. Consistent with this view, both SGK and PKBα served as substrates for and were activated by yeast Pkh1. Conversely, Ypk1 was a good substrate for and was activated by PDK1. Moreover, Pkh1-activated Ypk1 had a similar substrate specificity *in vitro* to that of PDK1-activated PKBα and SGK, at least on peptide substrates. Furthermore, we showed that Pkh1 is able to phosphorylate and activate Ypk1 *in vitro*, is required for optimal phosphorylation of Ypk1 *in vivo*, and, when overproduced, is able to stabilise a temperature-sensitive Ypk1 mutant. Mutagenesis studies indicated that Pkh1 phosphorylates Ypk1 at Thr504, which resides in a context equivalent to Thr308 in PKBα and Thr256 in SGK, the sites phosphorylated by PDK1 (and Pkh1). Collectively, these results indicate that Ypk1 (and, most likely, Ykr2) lie downstream of Pkh1 (and/or Pkh2) in a protein kinase cascade that is essential for both the growth and survival of yeast cells.

PDK1 seems to be responsible for the activation of a number of different classes of signal-transducing protein kinases. Likewise, in yeast, evidence suggests that Pkh1 and Pkh2 may have other targets in addition to Ypk1 and Ykr2. First, human PKBα can be partially activated by a T308D mutation (in the PDK1 site) or by a S473D mutation (in the PDK2 site), and almost fully activated by both mutations [24]; nonetheless, expression of PKBα(T308D S473D), with or without its PH domain, is unable to support sustained vegetative growth of either *pkh1Δ pkh2Δ* or *ypk1Δ ykr2Δ* cells (A.C, and P.D.T., unpublished observations). Second, high levels of overexpression (~50-fold) of either Ypk1 or Ykr2 from the *GAL1* promoter on a multi-copy plasmid does not bypass the need for Pkh1 and Pkh2 function (P.D.T., unpublished observations), suggesting that Pkh1 and/or Pkh2 have other substrates essential for cell viability or that Ypk1 and Ykr2 possess no basal activity at all in the absence of Pkh1- and/or Pkh2-dependent phosphorylation. As we have described here, mutation of Thr504 or Thr662 (or both) to aspartic acid residues does not generate constitutively active Ypk1; likewise, SGK(T256D S422D), which contains the equivalent mutations, is also not constitutively active [23]. Hence, we do not know if constitutive activation of Ypk1 (or SGK) would be sufficient to rescue the lethality of a *pkh1Δ pkh2Δ* double mutant. A third reason to suspect that Pkh1 and Pkh2 have multiple essential targets is that a potential substrate, Pkc1, is necessary for yeast cell viability under standard growth conditions [37]; yet, overexpression of *PKC1* does not rescue the lethality of *pkh1Δ pkh2Δ* cells (A.C., unpublished observations). Likewise, absence of another potential Pkh1 and Pkh2 target, Sch9, which serves as an effector enzyme in a pathway parallel to (and largely redundant in function with) the three yeast PKA catalytic subunits (Tpk1, Tpk2 and Tpk3) [36,49], is sufficient to cause very slow growth. Tpk1, Tpk2 and Tpk3,

which comprise an essential gene set [36], may themselves be substrates for Pkh1 and/or Pkh2 as all three possess a PDK1-like phosphorylation site [22].

Like mammalian PDK1 targets, Ypk1 and other suspected substrates for yeast Pkh1 and Pkh2 (except Tpk1, Tpk2 and Tpk3) contain the consensus PDK2 sequence (Phe–X–X–Ar–**Ser/Thr**–Ar, where the bold residue is the phosphorylated amino acid and Ar represents an aromatic residue) located at about the same position (160–165 residues carboxy-terminal) relative to the threonine residue phosphorylated by Pkh1. This conservation strongly suggests that *S. cerevisiae* possesses a PDK2-like activity that may act coordinately with Pkh1 and Pkh2 to modulate the state of activation of a number of target protein kinases, as observed for PDK1 and PDK2 in animal cells.

Since the submission of this manuscript, the identification of the *Schizosaccharomyces pombe* gene *ksg1* has been published. The *ksg* gene has structural homology with *PKH1*/*PKH2* and is also essential for growth, but is not essential for spore germination [50].

Materials and methods

Gene disruptions and strain constructions

A PCR-based method [51] was used for disruption of the *PKH1* and *PKH2* genes. The *HIS3* marker was obtained from pRS313, and the *TRP1* marker from pRS314 [52]. The *ypk1-Δ1::HIS3* allele and the *ykr2-Δ1::TRP1* alleles [45] will be described in greater detail elsewhere (P.D.T., E.A. Schnieders and J.T., unpublished data); in both mutations, the coding sequences for the entire catalytic domains of both enzymes were deleted and replaced with the indicated markers. To generate a *ypk1Δ ykr2Δ* double mutant, a *MATα ypk1Δ* strain (YES5) was mated to a *MAT***a** *ykr2Δ* strain (YES1) carrying p*YKR2*(*URA3*) (see below). The resulting diploid was subjected to sporulation and tetrad dissection, and a $His^+Trp^+Ura^+$ spore, representing a *MAT***a** *ypk1Δ ykr2Δ* cell kept alive with the plasmid-borne *YKR2* gene, was designated YPT28. To create the temperature-conditional *ypk1-1^{ts} ykr2Δ* strain, a temperature-sensitive allele was first generated by error-prone PCR amplification of *YPK1* DNA [33]. The linear PCR products were cloned by a procedure that allows for substitution of the potentially mutant *YPK1* sequences into a gapped vector, regenerating circular plasmids via recombination with homologous sequences present at each end of the PCR product [53,54], and scored for the desired phenotype by a plasmid shuffle procedure [55]. The desired mutant plasmid was recovered [56] and used to construct a *HIS3*-marked derivative that was integrated in place of the chromosomal *YPK1* locus in a *ykr2Δ* strain, yielding YPT40.

Plasmids

Plasmids for expression of *PKH1*, *YPK1* and *SGK*, as GST fusions in mammalian 293 cells, were constructed in the vector pEBG-2T [57]. Plasmids for the expression of *PKH1* in yeast were constructed in vectors YEplac195 [58] and pYES2 [59]. The vector pYES2 was used to express *PKH2* under the control of the *GAL1* promoter. To express mammalian PDK1 in yeast under the control of the *PKH1* promoter, the *PKH1* promoter region was amplified by PCR, fused to a fragment comprising the amino-terminal sequence of PDK1, and the resulting construct used to replace the corresponding segment in plasmids containing either intact PDK1, yielding YEplac195-PDK1, or a derivative of PDK1 lacking its carboxy-terminal PH domain, yielding YEplac195-PDK1-ΔPH. The vector pYES2 was used to express genes encoding human PDK1 and PDK1-ΔPH under control of the *GAL1* promoter. The vector YEp351GAL [60] was used to express *YPK1* and *YKR2* in yeast under control of the *GAL1* promoter. Alternatively, an HA-tagged version of the *YPK1* coding sequence, generated by PCR, was inserted into pYES2. A PCR-based method for precise gene fusion [61] was used to generate a Ypk1 derivative tagged at its carboxy-terminal end with the c-Myc epitope recognised by the monoclonal antibody 9E10 [62]. Low-copy-number (CEN) vector pRS316 [51] carrying the *URA3* gene was used to express *YKR2* under control of its endogenous promoter. Appropriate constructs to express the mammalian protein kinases, rat SGK, mouse PKBα, mouse p70 S6 kinase, and bovine βARK, were constructed in the vectors, pAD4M [63] and YEp351GAL [60], as indicated in the text. The vector pYES2 was used to express human PKBα [24] and a constitutively active variant of PKBα in which Thr308 and Ser473 were replaced by aspartic acid.

Site-directed mutagenesis

To generate a catalytically inactive (kinase-dead) version of Pkh1 (Pkh1(D267A)), Asp267 was changed to Ala. This position corresponds to a conserved residue critical for recognition of the Mg^{2+}–ATP substrate in all protein kinases [8]. Likewise, a catalytically inactive Ypk1 derivative was generated by changing Asp488 (nucleotide sequence GAT) to Ala (GCT). To attempt to generate a constitutively active YPK1 derivative, the Pkh1-dependent phosphorylation site (Thr504) and a presumptive phosphorylation site (Thr662) that matches the consensus for phosphorylation by a PDK2-like enzyme were replaced with aspartic acid.

Expression and purification of GST–Pkh1, GST–Ypk1 and GST–SGK

The 293 human embryonic kidney cell line was cultured on 40 10-cm diameter dishes, and each dish was transfected with 20 μg of the appropriate expression construct using a modified calcium phosphate method [64]. Lysates were prepared and the resulting GST fusion proteins were purified as described previously for GST–PKBα [3]. About 0.5 mg of each purified GST fusion protein was obtained, snap-frozen in aliquots in liquid nitrogen and stored at –80°C.

Measurement of Ypk1, SGK and PKB activities

The assay of Ypk1 activity was carried out in two stages. First (stage 1), GST–Ypk1 was activated by incubation with GST–Pkh1 and Mg^{2+}–ATP, as follows. A reaction mixture (18 μl) containing 2.5 μM PKI, 1 μM microcystin-LR, 10 mM Mg–acetate, 100 μM unlabelled ATP and 0.6 μM GST-Ypk1 was prepared in buffer A (50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, and 0.1% (by vol) 2-mercaptoethanol). The reaction was initiated by addition of 2 μl 50 nM GST–Pkh1 in buffer A containing 1 mg/ml bovine serum albumin, and was incubated at 30°C for 30 min. Second (stage 2), activated Ypk1 was assayed by adding 30 μl of a mixture in buffer A containing 2.5 μM PKI, 1 μM microcystin-LR, 10 mM Mg–acetate, 100 μM [γ^{32}P]ATP (200–400 cpm/pmol) and 100 μM crosstide (GRPRTSSFAEG in single-letter amino-acid code) [32], a peptide phosphoacceptor substrate. After incubation for 15 min at 30°C, the reaction was terminated by spotting a portion (45 μl) of each reaction mixture onto small squares of phosphocellulose paper (Whatman P81), which were washed and analysed as described [65]. Control reactions omitted either GST–Ypk1 or GST–Pkh1 and resulted in incorporation of less than 5% of the radioactivity measured in the presence of both of these proteins. One unit of GST–Ypk1 activity was defined as that amount required to catalyse phosphorylation of 1 nmol crosstide in 1 min. Assay of SGK and PKBα activities were carried out in identical manner, except that GST–SGK and GST–PKBα, respectively, replaced GST–Ypk1 in the first stage of the assay.

Phosphorylation of GST–Ypk1, GST–PKBα and GST–SGK by Pkh1

Incubations were identical to stage 1 of the Ypk1 assay described above, except that [γ-^{32}P]ATP (500–1,000 cpm/pmol) was used instead of unlabelled ATP, and reactions were terminated by adding SDS to a final concentration of 1%. The resulting samples were

resolved on 7.5% SDS–polyacrylamide gels [66] and, after staining with Coomassie blue, analysed by autoradiography. Also, the stained band corresponding to the GST fusion protein of interest was excised and the amount of radioactivity incorporated was quantified by liquid scintillation counting. Ability of GST–Pkh1 to phosphorylate and activate human SGK was examined using methods identical to those described immediately above. For phosphorylation and activation of human PKBα by GST–Pkh1, reactions were performed in the presence of lipid vesicles containing various 3-phosphoinositides [3], as described in the Results. Lipid abbreviations: SA-PI(3,4,5)P_3 is the D-enantiomer of *sn*-1-stearoyl, 2-arachidonyl PI(3,4,5)P_3; DP-PI(3,4,5)P_3 is *sn*-1,2-dipalmitoyl D-PI(3,4,5)P_3; DP-PI(3,4)P_2 is *sn*-1,2-dipalmitoyl D-PI(3,4)P_2; DP-PI(3)P is *sn*-1,2-dipalmitoyl D-PI(3)P. PI(4,5)P_2 was purified from brain extract.

Determination of Ypk1 substrate specificity

GST–Ypk1 was activated with Pkh1 *in vitro* and incubated under standard assay conditions, as described above, except that crosstide was replaced by 100 μM of the peptides discussed in detail in the Results. GST–PKBα and GST–SGK, activated by phosphorylation with PDK1 *in vitro* were assayed in parallel.

Supplementary material

A figure showing the *in vivo* Pkh1-dependent phosphorylation of Ypk1, a table of the yeast strains used in this study and additional methodological details are published with this paper on the internet.

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Supplementary material

Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast

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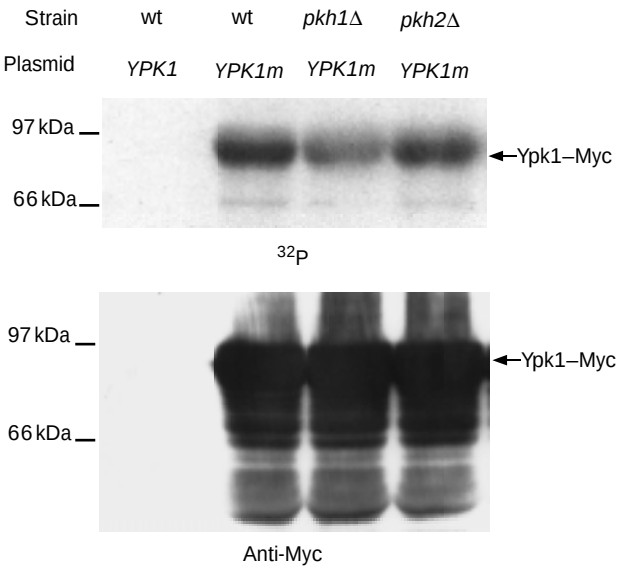
Figure S1



Phosphorylation of Ypk1 *in vivo* is dependent on Pkh1. Haploid strains AC301 (*pkh1Δ*) and AC303 (*pkh2Δ*), and their otherwise isogenic parent (wt), were transformed, as indicated, with either a vector expressing *YPK1* or the same vector expressing Ypk1 tagged with a c-Myc epitope (*YPK1m*) and metabolically labelled with $[^{32}P]PO_4^{3-}$. Whole-cell extracts were prepared, clarified, and then subjected to immunoprecipitation with mouse anti-c-Myc monoclonal antibody 9E10. After extensive washing, duplicate samples of the immunoprecipitates were solubilised, subjected to SDS–PAGE, transferred to a PVDF membrane, and analysed by autoradiography to measure ^{32}P incorporated (upper panel) and by immunoblotting with the anti-Myc monoclonal antibody (lower panel) to assess the relative recovery of Ypk1–Myc in each sample.

Supplementary materials and methods

Cell culture

S. cerevisiae strains used are described in Table S1, and were derivatives of either AYS927 (W303 background) or YPH499 (S288C background). Yeast was grown at 30°C in a rich medium (YPD) containing 1% yeast extract, 2% peptone (Difco) and 2% glucose, or in a synthetic minimal medium (S), containing either galactose (Gal), raffinose (R) or glucose (Glc, or D, for dextrose) as the carbon source, supplemented with nutrients appropriately to maintain selection for markers and plasmids [S1]. Standard methods were used for yeast genetic manipulation [S1,S2] and for plasmid construction and propagation in *Escherichia coli* [S3].

Recombinant DNA techniques

Restriction enzyme digests, DNA ligations and other recombinant DNA procedures were performed as described [S3]. Modification of the Li–acetate procedure was used for yeast transformation [S4]. *PKH1* and *PKH2* genes used in this study, and the *YPK1* gene in some constructions, were recovered from genomic DNA of strain AYS927 using PCR amplification, gel-purified, and cloned first into the pCR2.1-TOPO vector (Invitrogen). Site-directed mutagenesis was performed using the QuikChange Kit (Stratagene) following instructions provided by the manufacturer. DNA constructs were verified by automatic DNA sequencing using an automated DNA Sequencer (Model 373; Applied Biosystems).

PCR amplification of yeast genomic DNA

The primers used to amplify the *PKH2* coding region were 5′-CG*GGATCC*GCCACCATGGAGCAGAAGCTGATCTCTGAAGAGGACTTGTATTTGATAAGGATAATTCCATG-3′ (forward) and 5′-ATAAGAAT*GCGGCCGC*TTACGACCTCTTCGATTTTGCAG-3′ (reverse), and incorporated *Bam*HI and *Not*I sites, respectively (indicated by italics). The primers used to amplify the *PKH1* coding region were 5′-ATAAGAAT*GCGGCCGC*TGCCACCATGGAGCAGAACCTGTCTCTGAAGAGGACTTGGGAAATAGGTCTTGACAGAGG-3′ (forward) and 5′-ATAAGAAT*GCGGCCGC*TCATTTTTCATCTGTCCGTGTC-3′ (reverse), and incorporated *Not*I sites (indicated in italics). Both 5′ primers also contained a sequence encoding a 10-residue c-Myc epitope tag (underlined). The primers used to amplify the *YPK1* gene were: 5′-*GGATCC*GCCACCATGTACCCATACGATGTGCCAGATTACGCCTATTCTTGGAAGTTTAAG-3′ (forward) and 5′-*GGTACC*CTATCTAATGCTTCTACCTTGC-3′ (reverse), and incorporated *Bam*HI and *Kpn*I restriction sites, respectively (italics). The initiator or termination codons in all these primers are also indicated (bold type).

PCR-based gene disruptions and strain constructions

To generate the *pkh2Δ::HIS3* mutation, *HIS3* was amplified from pRS313 [S5] using as primers 5′-AAGTAACATCTTGATGAACCGAGAAGCCACTAACTAGTTTTGTGCACCATAATTTTCCG-3′ (forward), where the underlined sequence corresponds to nucleotides –93 to –53 from the *PKH2* initiator codon and the remainder of the primer corresponds to nucleotides –326 to –311 from the initiator codon of *HIS3*, and 5′-TAAGTAGCTTGATGAAAACATTAGATAAAATTACTAATTACCGTCGAGTTCAAGAG-3′, (reverse) where the underlined sequence corresponds to the nucleotides immediately after the *PKH2* stop codon (in bold type) and the remainder corresponds to nucleotides 204–189 after the *HIS3* stop codon. The resulting 3.3 kb product was used for DNA-mediated transformation of a diploid strain (AYS927). Transformants were selected on SD-His plates, and disruption was verified by PCR analysis of DNA from one of the His^+ isolates using appropriate primers. This heterozygous *PKH2/pkh2Δ::HIS3* diploid (AC200) was sporulated, and the resulting tetrads were dissected. Spore clones were analysed by plating on selective medium and confirmed by PCR to identify a haploid containing the *pkh2Δ::HIS3* disruption (AC303). To generate the *pkh1Δ::TRP1* mutation, the *TRP1* marker in pRS314 [S5] was amplified using 5′-GCACGTGTACTTGCTTGAATACTGCTACTATATCATTAATATGGTACTGAGAGTGCACC-3′ (forward), where the underlined sequence corresponds to nucleotides immediately upstream of the initiator codon (bold type) and the remainder corresponds to nucleotides situated –300 to –285 nucleotides from the initiator codon of *TRP1*, and 5′-TATTATGCATTACACTTTCCCCTTCACCATGTCTTACATATGCATCCGCAGGCAAGTGCAC-3′ (reverse), where the underlined nucleotides correspond to positions +69 to 25 after the *PKH1* stop codon and the remainder of the primer corresponds to the region situated +51 to 36 nucleotides after the *TRP1* stop codon. The resulting

Table S1

***S. cerevisiae* strains used in this study.**

Strain	Genotype	Source
AYS927	*MAT*a/*MAT*α *ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 can1-100/can1-100 ssd1-d2/ssd1-d2*	M.J.R. Stark
AC200	AYS927 *PKH2/pkh2Δ::HIS3*	This study
AC201	AYS927 *PKH1/pkh1Δ::TRP1*	This study
AC303	*MAT*a *pkh2Δ::HIS3* (derived from AC200)	This study
AC301	*MAT*α *pkh1Δ::TRP1* (derived from AC201)	This study
AC306	*MAT*a/*MAT*α *PKH1/pkh1Δ::TRP1 PKH2/pkh2Δ::HIS3* (AC303 X AC301)	This study
YPH499	*MAT*a *ade2-101^{oc} his3-Δ200 leu2-Δ1 lys2-801^{am} trp1-Δ1 ura3-52*	[S5]
YPH500	*MAT*α otherwise isogenic to YPH499	[S5]
YES1	YPH499 *ykr2-Δ1::TRP1*	This study
YES5	YPH500 *ypk1-Δ1::HIS3*	This study
YES7	*MAT*a/*MAT*α *YPK1/ypk1Δ::HIS3 YKR2/ykr2Δ::TRP1* (YES1 X YES5)	This study
YPT28	*MAT*a *ypk1Δ::HIS3 ykr2Δ::TRP1* [p*YKR2*(*URA3*)]	This study
YPT40	*MAT*a *ypk1-1^{ts} ykr2Δ*	This study

2.4 kb product was used for transformation of AYS927, and transformants were selected on SD-Trp plates. Disruption was verified by PCR analysis from one of the Trp$^+$ isolates using appropriate primers. This heterozygous diploid *PKH1/pkh1Δ::TRP1* (AC201) was sporulated, dissected, analysed, and a haploid spore containing the *pkh1Δ::TRP1* mutation (AC301) was identified.

Generation of the ypk1-1^{ts} ykr2Δ *strain*

To create the temperature-conditional *ypk1-1^{ts} ykr2Δ* strain (YPT40), a temperature-sensitive allele of *YPK1* was first generated, as follows. A genomic insert containing the *YPK1* gene cloned into the *Xba*I site in the vector, pGEM3^{TM} (Promega), and generously provided by Richard A. Maurer (then at the University of Iowa) [S6], was excised as a 4.1 kb *Xba*I–*Sal*I fragment and inserted into the *LEU2*-containing vector, pRS315 [S5], yielding pRS315-*YPK1*. The sequence encoding the catalytic domain of *YPK1* was then amplified under moderately error-prone conditions using AmpliTaq^{TM} DNA polymerase (Perkin Elmer Cetus), with pRS315-YPK1 as the template and a 5′ primer (P1), 5′-TGCCCTCGAAGACATGGC-3′, corresponding to a sequence beginning at nucleotide 788 (where the first base of the ATG start codon is +1) and a 3′ primer (P2), 5′-CTTGAACACAGTAAGTAACGG-3′, corresponding to the flanking genomic sequence commencing 68 bp downstream of the stop codon. The resulting 1350 bp linear PCR product was gel-purified and co-transformed into a haploid *ypk1Δ ykr2Δ* double mutant carrying p*YKR2*(*URA3*) (strain YPT28) along with a ~9 kb linear fragment of pRS315-*YPK1*, that had been generated by digestion with *Pst*I and *Nco*I, and gel-purified. Transformants were selected on SCD-Leu plates at 26°C. This procedure allows for replacement of the corresponding sequence in the parent vector with potentially mutant sequences, and regeneration of circular plasmids, via *in vivo* repair of the gapped plasmid by recombination with homologous sequences present at each end of the PCR product [S7,S8].

To determine which *LEU2*-containing plasmids expressed functional *YPK1* at 26°C, the Leu$^+$ transformants were subsequently replica-plated onto Leu$^-$ plates containing 5-FOA to select for loss of the p*YKR2*(*URA3*) plasmid initially present in YPT28. To determine which of the *YPK1*- and *LEU2*-containing plasmids harboured a temperature-sensitive allele of *YPK1*, the Leu$^+$ Ura$^-$ cells were tested by replica-plating for their ability to grow on SCD-Leu plates at 37°C. One transformant was identified that reproducibly failed to grow at this temperature. The *LEU2*-containing plasmid carried by this strain (*pypk1TS*) was recovered [S9], and direct nucleotide sequence analysis of the *YPK1* open reading frame in the plasmid revealed the presence of two amino acid substitutions (I484T and Y536C). Subcloning and re-transformation confirmed that these mutations were sufficient to confer the temperature-sensitive (ts) phenotype, and this allele was designated *ypk1-1^{ts}*. The *ypk1-1^{ts}* allele was used to transplace the normal *YPK1* chromosomal locus in the *ykr2Δ* strain (YES1), as follows. First, PCR was used to generate a customised DNA fragment containing *Bam*HI and *Sma*I restriction sites 96 and 108 bp, respectively, downstream from the stop codon of the *YPK1* coding sequence in *pypk1TS* plasmid, and this fragment was substituted for the corresponding segment of the 3′-flanking region, yielding pINT. A 2.6 kb *Sca*I–*Bam*HI fragment containing the *HIS3* gene, excised from vector, pRS303 [S5], was gel-purified and inserted into pINT that had been digested with *Bam*HI and *Sma*I, yielding pINT-HIS, which was able to confer both leucine and histidine prototrophy to a *leu2 his3* strain, YPH499 [S5]. Finally, a 4.6 kb *Cla*I–*Xho*I fragment from pINT-HIS, containing the *ypk1-1^{ts}* allele, the *HIS3* gene, and additional genomic DNA from the *YPK1* locus flanking the *HIS3* gene to its 3′-side, was gel-purified and used for transformation of YES1. His$^+$ transformants were selected at 26°C, The presence of the integrated *ypk1-1^{ts}* allele (and the absence of the normal *YPK1* locus) was then confirmed by PCR analysis of DNA isolated from the transformants and by demonstrating that such cells were unable to grow at 37°C. One such isolate that met all of these criteria was designated strain YPT40.

Plasmids

For expression of *PKH1* and *YPK1* as GST fusions in mammalian 293 cells, the corresponding coding sequences were excised from the

appropriate pCR2.1-TOPO derivative and inserted into the mammalian expression vector, pEBG-2T [S10]. PKBα [S11], PDK1 [S12] and SGK (lacking its amino-terminal 60 residues) [S13] were subcloned into the pEBG-2T vector as described. For expression of *PKH1* in yeast, a 2.1 kb fragment, from an internal *Sma*I site (situated about 250 bp downstream from the initiator codon) to an *Eco*RI site in the pCR2.1-TOPO vector, was first inserted into the 2 μm DNA vector, YEplac195 [S4], that had been digested with *Sma*I and *Eco*RI, yielding YEplac195-2.1*PKH1*. To restore the 5′ end, a 1 kb fragment was amplified by PCR using the primers 5′-GCTTGACTCAATTAAGGC-GAC-3′ (forward), corresponding to nucleotides 628–634 upstream of the initiator codon, and 5′-ACATGCTTAGTTAACTCC-3′ (reverse), corresponding to the region located 350 bp downstream of the initiator codon. The resulting product was first cloned into pCR2.1-TOPO, which was then digested with *Sma*I and *Sph*I to liberate a 0.9 kb fragment that was inserted into the YEplac195-2.1*PKH1* construct that had been digested with *Sph*I and *Sma*I, yielding YEplac195-*PKH1*, which contains the complete coding region of the *PKH1* gene plus 0.5 kb of its promoter region and carries the *URA3* gene as the selectable marker. To express *PKH1* under control of the *GAL1* promoter, a 2.3 kb *Not*I–*Not*I fragment containing the Myc-tagged version of the entire *PKH1* coding sequence was inserted into the *URA3*-marked, 2 μm DNA-containing vector, pYES2 [S14], yielding pYES2-*PKH1*. Likewise, to express *PKH2* under *GAL1* promoter control, a 3.3 kb *Bam*HI–*Not*I fragment containing the entire *PKH2* open reading frame was inserted into pYES2, yielding pYES2-*PKH2*.

To express mammalian PDK1 in yeast under control of the *PKH1* promoter, a 0.7 kb fragment corresponding to the *PKH1* promoter region was first amplified by PCR from the YEplac195-*PKH1* construct using the primers 5′-GG*GGTACC*GCTTGACTCAATTAAGGCGAC-3′ (forward) and 5′-CTTCAGAGATCAGCTTCTGCTCCATATTAAT-GATATAGTA-3′ (reverse), corresponding to the start of the PDK1 coding sequence (underlined). Second, a 1.4 kb fragment comprising the amino-terminal sequence of PDK1 was amplified from a human PDK1 cDNA using as primers the 0.7 kb PCR amplification product (forward) and 5′-ACACGATCTCAGCCGTGTAAA A-3′(reverse), corresponding to residues 190–184 of PDK1. The 1.4 kb product was cleaved at the *Kpn*I site (italics) and also with *Hind*III, which cleaves at an internal site in the *PDK1* coding sequence. The resulting *Kpn*I–*Hind*III fragment was used to replace a 0.5 kb segment encoding the amino-terminal end of the PDK1 protein either in a construct containing the complete PDK1 coding sequence, yielding YEplac195-PDK1, or in a construct containing just the first 404 residues of the PDK1, corresponding to its catalytic domain and lacking its carboxy-terminal PH domain, generating YEplac195-PDK1-ΔPH. To express human PDK1 under control of the *GAL1* promoter, a 2.0 kb *Bgl*II–*Xba*I fragment containing the complete PDK1 coding sequence was inserted into pYES2, yielding pYES2-PDK1. Similarly, a 1.4 kb *Bgl*II–*Xba*I fragment containing the kinase domain of PDK1, but lacking the PH domain, was inserted into pYES2, creating pYES2-PDK1-ΔPH.

To express *YKR2* in yeast from a *LEU2*-marked, high-copy-number (2 μm DNA-based) plasmid under control of the *GAL1* promoter, a 2.4 kb *Xho*I–*Hind*III fragment of genomic DNA containing the entire *YKR2* open reading frame [S15] was excised from an insert in pUC18 (generously provided by Shigeo Ohno, Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo) and ligated into the vector, YEp351GAL [S16], that had been linearised by digestion with *Sal*I and *Hind*III, yielding pGAL-*YKR2*. An essentially identical approach was used to express *YPK1*, excised from a genomic DNA fragment (see above), yielding pGAL-*YPK1*, which was constructed by Henrik Dohlman (Thorner laboratory). Alternatively, a 2.1 kb *Bam*HI-*Not*I fragment encoding an HA-tagged version of the *YPK1* coding sequence, generated by PCR, was inserted into pYES2, yielding pYES2-*YPK1*. To place the *YKR2* gene under control of its endogenous promoter on a low-copy-number (CEN) plasmid carrying the *URA3* gene, a 2.5 kb *Eco*RI–*Eco*RI fragment of the original *YKR2*-containing insert in pUC18 was ligated into the vector, pRS316 [S5], that had been linearised with *Eco*RI, generating p*YKR2*(*URA3*). To generate a version of Ypk1 tagged at its carboxyl terminus with the c-Myc epitope recognised by the monoclonal antibody 9E10 [S17], a PCR-based method for precise gene fusion [S18] was performed using the *YPK1* sequence cloned in pGEM3 as one template, and, as the other template, a sequence encoding the 16-residue version of the Myc epitope followed by a His_6 tag cloned in pBluescript (Stratagene), generously provided by Elana Swartzman (Thorner laboratory), and three appropriate synthetic oligonucleotide primers: P1; T3 (Stratagene), 5′-AATTAACCCTCACTAAAGGG-3′, corresponding to sequences in the pBluescript vector; and, a 'joiner' primer (P3), 5′-TTCAGAAATCAACTTTTGTTCTCTAATGCTTCTACCTTGC-3′, corresponding to the 3′-end of the *YPK1* coding sequence and the first several residues of the c-Myc epitope. A 2 kb *Cla*I–*Sal*I fragment of the resulting product was used to replace the corresponding segment in the original *YPK1*-containing pGEM3 vector [S6], yielding p*YPK1myc*. A 1.2 kb *Nco*I–*Hind*III fragment of p*YPK1myc* was used to replace the corresponding segment of pGAL-*YPK1*, yielding pGAL-*YPK1myc*.

To express *SGK* in yeast, a 1.3 kb *Nco*I–*Eco*RI fragment encoding a rat *SGK* cDNA [S19] (generously provided by Gary Firestone, Department of Molecular and Cell Biology, University of California, Berkeley, California) was converted to blunt ends by treatment with the Klenow fragment of *E. coli* DNA polymerase I in the presence of dNTPs and inserted behind the *ADH1* promoter in the vector pAD4M [S20] that had been linearised with *Sma*I. Correct orientation of the fragment was confirmed by appropriate restriction enzyme digests. An essentially identical approach was used to express bovine βARK, yielding pADH-*βARK*, which was constructed by Henrik Dohlman (Thorner laboratory). To express PKB in yeast, a 1.5 kb *Bam*HI–*Bam*HI fragment encoding mouse c-Akt was excised from an insert in a two-hybrid bait vector, pASIIA (supplied by Zhou Songyang, Department of Biology, Massachusetts Institute of Technology, Cambridge), and ligated into YEp351GAL that had been linearised by digestion with *Bam*HI, yielding pGAL-*PKB*. Alternatively, a 2.5 kb *Eco*RI–*Xba*I fragment encoding a human PKBα cDNA [S21] was inserted into pYES2, generating pYES2-PKBα. In addition, a 1.5 kb *Eco*RI–*Xba*I insert expressing a constitutively active mutant version of PKBα [S22], in which Thr308 and Ser473 have been replaced by aspartic acid residues, was inserted into pYES2, creating pYES2-DD-PKBα. To express p70 S6K in yeast, a 1.6 kb *Xba*I–*Sal*I fragment encoding rat p70 S6K [S23] was excised from p2B4 (kindly provided by George Thomas, Friedrich Miescher Institute, Basel, Switzerland) and inserted into YEp351GAL that had been linearised by digestion with *Xba*I–*Sal*I, yielding pGAL-*S6K*. In addition, an amino-terminal truncation, a carboxy-terminal truncation, and a double amino- and carboxy-terminal truncation of p70 S6 kinase (generously provided by John Blenis, Department of Cell Biology, Harvard Medical School, Boston), whose constructions are described in detail elsewhere [S24], were also inserted into a yeast expression vector, YEp352 [S25], each under control of the methionine-repressible *MET3* promoter [S26] using essentially identical methods.

Plasmid p*PKH1*-HA was constructed by PCR as follows: Primers 5′-TGCGCTCGAG*ATG*GGAAATAGGTCTTTG-3′ (underlined bases correspond to an introduced *Xho*I site, and start codon in italics) and 5′-CGCATGCA*TTA*GCGGCCGCCTTTTTCATCTGTCCGTG-3′ (underlined bases correspond to an introduced *Nsi*I site, italic bases the stop codon, and bold bases correspond to an introduced *Not*I site) were used to amplify the entire open reading frame of *PKH1* from pYES2-*PKH1*. This 2.2 kb PCR fragment was then digested with *Xho*I and *Nsi*I and ligated into vector YEp351GAL that had been digested with *Sal*I and *Pst*I. The resulting plasmid, p*PKH1*, contained a *Not*I site just before the stop codon to permit in-frame insertion of a fragment encoding three tandem HA epitope tags, which were excised as a *Not*I fragment from a plasmid described elsewhere [S27]. Orientation of the tag was confirmed by sequencing. Plasmid p*PKH2*-HA was constructed by PCR in a very similar manner, using primers 5′-TGCGGGATCC*ATGT*ATTTTGATAAGGATAAT-3′, (underlined bases correspond to an introduced *Bam*HI site, start codon in italics) and

5′-CCGCTCGAG*TTA*GCGGCCGCCCGACCTCTTCGATTTTG-3′ (underlined bases correspond to an introduced *Xho*I site, italic bases the stop codon, and bold bases correspond to an introduced *Not*I site) to amplify the entire open reading frame of *PKH2* from pYES2-*PKH2*. The 3.2 kb PCR fragment was then digested with *Bam*HI and *Xho*I and ligated to vector YEp351GAL that had been digested with *Bam*HI and *Sal*I.

Radiolabeling of yeast cells and immunoprecipitation of Ypk1 from cell extracts

Yeast strains harbouring pGAL-*YPK1myc* were grown at 30°C to mid-exponential phase in raffinose-containing minimal medium, supplemented with all L-amino acids and nucleotides (except Leu and Ura). The cells were collected, diluted into galactose-containing low phosphate medium [S28], grown to mid-exponential phase, collected, concentrated 10-fold and incubated for 2.5 h with 0.5–1 mCi of $^{32}PO_4^{3-}$. Radiolabeled cells were collected by brief sedimentation in a clinical centrifuge, washed with PBS and resuspended in ice-cold extraction buffer containing a mixture of phosphoprotein phosphatase inhibitors and protease inhibitors that has been described [S29]. Extracts were prepared and Ypk1–Myc protein was immunoprecipitated with the 9E10 anti-c-Myc monoclonal antibody, according to methods described in detail elsewhere [S30]. The final washed immune complexes were resuspended in 15 μl SDS–PAGE sample buffer, and heated to 90°C for 5 min. Samples were clarified by centrifugation for 2 min in a microfuge, and the supernatant solution (typically ~25 μl) was subjected to electrophoresis on a 8% polyacrylamide gel in the presence of SDS. Autoradiography of dried gels or PVDF filter replicas was performed using either X-ray film or a Phosphorimager (Storm™; Molecular Dynamics), according to procedures recommended by the manufacturer.

Antisera

A rabbit polyclonal anti-Ypk1 antiserum (#1446) was raised against a GST–Ypk1 fusion protein containing the first 115 residues of Ypk1, expressed in and purified from *E. coli*. For immunoblotting, the antiserum was used at 1:3,000 dilution. Other antisera used in this study were the gifts of other investigators.

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